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Aldosterone increases $K_{Ca}1.1$ (BK) channel-mediated colonic K^+ secretion

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Mammalian K^+ homeostasis results from highly regulated renal and intestinal absorption and secretion, which balances the unregulated K^+ intake. Aldosterone is known to enhance both renal and colonic K^+ secretion. In mouse distal colon K^+ secretion occurs exclusively via luminal $K_{Ca}1.1$ (BK) channels. Here we investigate if aldosterone stimulates colonic K^+ secretion via BK channels. Luminal Ba^{2+} and iberiotoxin (IBTX)-sensitive electrogenic K^+ secretion was measured in Ussing chambers. *In vivo* aldosterone was augmented via a high K^+ diet. High K^+ diet led to a 2-fold increase of luminal Ba^{2+} and IBTX-sensitive short-circuit current in distal mouse colonic mucosa. This effect was absent in BK α-subunit-deficient ($BK^{-/-}$) mice. The resting and diet-induced K^+ secretion was stimulated by luminal ionomycin. In $BK^{-/-}$ mice luminal ionomycin did not stimulate K^+ secretion. *In vitro* addition of aldosterone likewise triggered a 2-fold increase in K^+ secretion, which was inhibited by the mineralocorticoid receptor antagonist spironolactone and the BK channel blocker IBTX. Semi-quantification of mRNA from colonic crypts showed up-regulation of BK α- and β_2-subunits in high K^+ diet mice. The BK channel could be detected luminally in colonic crypt cells by immunohistochemistry. The expression level of the channel in the luminal membrane was strongly up-regulated in K^+-loaded animals. Taken together, these data strongly suggest that aldosterone-induced K^+ secretion occurs via increased expression of luminal BK channels.

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The colon contributes to K^+ homeostasis by either secretion or absorption of K^+ under various dietary, hormonal or diseased states (Kunzelmann & Mall, 2002). Normally, the distal mammalian colon displays net K^+ secretion (Binder & Sandle, 1994; Kunzelmann & Mall, 2002; Binder, 2003). In patients with end-stage renal disease faecal K^+ excretion is directly proportional to dietary K^+ intake (Hayes *et al.* 1967). This implies that colonic 'K^+ adaptation' makes a substantial contribution to K^+ homeostasis in this disease and underscores the importance of defining the elements of colonic K^+ handling. Colonic K^+ secretion occurs either via a paracellular or a transcellular route (Binder, 2003). The respective quantitative importance of these two K^+ secretory pathways has yet to be established. Transcellular K^+ secretion occurs according to the pump leak mechanism (Binder & Sandle, 1994). K^+ enters the cell from the blood side via the basolateral Na^+/K^+-ATPase and the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1). K^+ exits via luminal K^+ channels (Pacha *et al.* 1987; Sweiry & Binder, 1989; Binder & Sandle, 1994). Previous studies had suggested luminal BK channels as the K^+ exit pathway in colonic and renal epithelia (Hunter *et al.* 1984; Butterfield *et al.* 1997). Recently, we have identified that BK channels indeed constitute the functionally relevant and only luminal K^+ pathway for distal colonic K^+ secretion (Sausbier *et al.* 2006). In the preceding study, Ussing chambers were used to measure ion transport in mice deficient in the BK channel α-subunit ($BK^{-/-}$). $BK^{-/-}$ mice displayed a significant colonic epithelial phenotype with: (1) lack of Ba^{2+}-sensitive resting K^+ secretion; (2) absence of K^+ secretion stimulated by luminal P2Y receptors; (3) absence of luminal Ca^{2+} ionophore-stimulated K^+ secretion; and (4) reduced K^+ and increased Na^+ contents in the faeces. BK channels were shown to localize to the luminal membrane of colonic enterocytes. RT-PCR results confirmed the expression of the BK channel α-subunit in isolated distal colonic crypts (Sausbier *et al.* 2006).

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Colonic K^+ secretion is known to be enhanced by aldosterone (Pacha *et al.* 1987; Sweiry & Binder, 1989; Rechkemmer & Halm, 1989; Halm & Halm, 1994); its mechanism, however, remains incompletely defined. A likely cause of aldosterone-stimulated colonic K^+ secretion could be the functional increase of the luminal K^+ exit pathway. This could either be mediated by the luminal BK channel itself, or by introduction of an alternative luminal K^+ conductance. We therefore used our $BK^{-/-}$ mouse to investigate if these animals treated on a high K^+ diet up-regulate an alternative Ba^{2+}-sensitive luminal K^+ conductance. It is established that a high K^+ diet dramatically increases plasma aldosterone in normal mice and especially $BK^{-/-}$ mice (Rieg *et al.* 2007). We find here that $BK^{-/-}$ mice on a high K^+ diet develop no electrogenic Ba^{2+}-sensitive colonic K^+ secretion, despite a dramatically elevated plasma aldosterone (Rieg *et al.* 2007). Thus, it is very likely that a high K^+ diet induces distal colonic K^+ secretion via the BK channel. Here we also investigate if the BK channel mediates the increased colonic K^+ secretion in mice fed a high K^+ diet and in isolated tissue exposed to exogenous aldosterone. For this purpose we searched for a suitable mouse strain with a strong biological responsiveness to endogenous aldosterone. We used the established colonic epithelial aldosterone-responsive gene *CHIF/FYXD4* (Wald *et al.* 1996) to quantify its expression in the NMRI outbred mouse strain and in the inbred B57/Bl6 mouse strain. We found that a high K^+ diet strongly up-regulates aldosterone and CHIF in the NMRI mouse strain and to a much lesser degree in B57/Bl6 mice (see Fig. 1 in online Supplemental material). Therefore, we supposed the NMRI mice to be a suitable mouse model to investigate the role of aldosterone on BK channel-mediated distal colonic K^+ secretion. We find that aldosterone activates K^+ secretion via increased expression and function of luminal BK channels.

Methods

Mice

NMRI wild-type (WT) mice were obtained from Taconic. They were subsequently bred in-house. Experiments were performed on age-matched animals (2–4 months old) of either sex. $BK^{-/-}$ and $BK^{+/+}$ on a SV129 × C57Bl6 hybrid background (F2 generation) (Sausbier *et al.* 2004) were bred in the Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Tübingen, Germany. Mice were kept on two different diets both from Altromin (Lage, Germany): a standard diet (1310) with a K^+ content of 10 g kg^{-1} and a high K^+ diet (C1050) with a K^+ content of 50 g kg^{-1} for a period of 3–5 days. Experiments were performed according to the Danish legislation on the protection of animals.

Serum aldosterone determinations

Blood sampling for serum aldosterone measurements was performed at 11.00 h in a group of control animals and a group of animals kept on the high K^+ diet for 4 days. The animals were anaesthetized with a 3% isoflurane gas mixture, killed and completely drained of blood (approx. 1 ml). Blood was collected in Microtainers (Becton Dickinson) and the tubes were centrifuged at 12 000 *g* for 2 min to separate serum from the blood cells. The total serum aldosterone was assayed using a commercial radioimmunoassay (RIA, Coat-A-Count) kit (Siemens Medical Solutions Diagnostics, Ballerup, Denmark).

Ussing chamber experiments

Mice of either sex were killed by cervical dislocation. The distal 2 cm of the entire (non-stripped) colonic sheet was mounted in an Ussing chamber. The two halves of the chamber were continuously perfused by a bubble lift system. The solutions on the two sides were symmetrical and had the following composition (in mM): NaCl 120; $NaHCO_3$ 25; K_2HPO_4 1.6; KH_2PO_4 0.4; calcium gluconate 1.3; $MgCl_2$ 1; D-glucose 5; in addition 5 μM indomethacin. The reservoirs were bubbled with 5% CO_2 and 95% O_2 and kept at 37°C by water jackets. Initially, tetrodotoxin (TTX, 1 μM) was added to the serosal side to inhibit possible secretory activation by the enteric nervous system or other autonomous nerve cells. Subsequently, amiloride (100 μM) was added to the mucosal perfusate to abolish electrogenic Na^+ absorption via the epithelial Na^+ channel (ENaC).

The experiments with tissue from mice on control and high K^+ diets were performed in *open-circuit* mode using chambers with an aperture of 0.126 cm^2. The open circuit experiments in $BK^{-/-}$ mice fed a normal or a high K^+ diet (Fig. 2) were performed with an aperture area of 0.283 cm^2. The open-circuit recording is the standard method in our laboratory and has the advantage of leaving the tissue in its natural electrical status without applying external electrical forces. The transepithelial voltage (V_{te}) is given in reference to the serosal side. Transepithelial resistance (R_{te}) was calculated from the voltage deflections (ΔV_{te}) induced by short current pulses (25 μA, 0.6 s). These deflections were corrected by values obtained in an empty chamber. The equivalent short-circuit current (I_{sc}) was calculated by Ohm's law from V_{te}/R_{te}. The calculated I_{sc} changes were derived from peak values. After an equilibration period of 30 min K^+ channel activators or inhibitors were added luminally. The measurement of the effect of agonists or antagonists was read 4 min after their addition unless otherwise stated.

Experiments with exogenous aldosterone were performed in a setup where the transepithelial voltage was

clamped to zero (Model DVC-1000, dual voltage clamp, WPI, USA) and the short-circuit current was measured directly. The data were recorded with PowerLab 4/25 from ADInstruments and analysed with the Chart (version 5.1) program. The rationale for changing from open-circuit to direct short-circuit current measurements was to improve the signal-to-noise ratio because capacitance voltage deflections are avoided in short-circuit measurement mode. The Ussing chamber aperture area in the direct I_{sc} measurements was 0.283 cm^2. After mounting, the tissues were allowed to equilibrate for 30 min. Then, $BaCl_2$ (5 mM) was added to the luminal solution and the Ba^{2+}-sensitive I_{sc} quantified. The tissues were then incubated for another 2 h with a specified concentration of aldosterone. After the aldosterone-containing solution was gently washed out and a short re-equilibration period of 10 min, $BaCl_2$ (5 mM) was added once more to the luminal solution to quantify the Ba^{2+}-sensitive I_{sc}. In the experiments with spironolactone and iberiotoxin (IBTX) the antagonists were present during the entire aldosterone incubation period.

Note that the experiments in Figs 1 and 2 were performed in a small-aperture tissue holder (0.126 cm^2). In contrast, in Figs 3, 6 and 7 a large-aperture tissue holder (0.283 cm^2) was used. A smaller size aperture causes an underestimation of the true R_{te} values (significant edge leakiness of the tissue). This will lead to overestimation of the true equivalent I_{sc} values. Thus, the absolute sizes of the blocker-sensitive K^+ secretory conductances can only be compared within experimental series.

Crypt preparations

For preparation of colonic crypts a 2 cm piece of mouse distal colon was everted and rinsed with ice-cold Ca^{2+}-free Ringer-type solution with the following composition (in mM): NaCl 127; KCl 5; sodium pyruvate 5; D-glucose 5; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes) 10; ethylenediaminetetraacetic acid (EDTA) 5; $MgCl_2$ 1. Both ends were tied to obtain a sac preparation. This sac was filled with the same Ca^{2+}-free solution. The sacs were then incubated in the-above mentioned solution for 10 min at 37°C. Isolated crypts were obtained by shaking the sacs and this preparation thus comprised almost pure epithelial enterocytes. Approximately 100 crypts were collected from this preparation. Crypts were centrifuged for 1 min at 1520*g* and the pellet was immediately re-suspended in lysis buffer (RNeasy Mini Kit, Qiagen), vortexed and frozen in liquid nitrogen.

Semi-quantitative PCR analysis of BK channel α- and β-subunits

Semi-quantitative PCR analysis was used to investigate mRNA levels of the murine BK α- and -$\beta_{1–4}$-subunits. Total RNA was extracted from isolated colonic crypts (7 control and 7 K^+-loaded animals) with the RNeasy Mini Kit (Qiagen). To remove possible genomic DNA contamination, total RNA was treated with RNase-free DNase (Qiagen). RNA was quantified with a Quant-iT RiboGreen RNA reagent (Stratagene). cDNAs were then synthesized from 300 ng of total RNA using SuperScriptIII reverse transcriptase (Invitrogen) and random decamer primers according to the manufacturer's instructions. The primers were validated for non-specific products with SYBR Green. qPCR with TaqMan probes (5′-labelled FAM and 3′-labelled BHQ1) was performed with the ExTaq enzyme (Takara) on an Mx3000P Quantitative PCR System (Stratagene). All reactions were carried out in duplicates. For the β_1, β_3 and β_4 subunits cDNA from a mixture of brain, testis and kidney were used as positive controls. Sequences and final concentrations of primers and probes are shown in Table 1.

The cycling profile for each run was: 95°C 10 min; 45 cycles 95°C 15 s followed by 60°C for 1 min. The expression of genes of interest was analysed in reference to the endogenous reference genes hypoxanthine-guanine phosphoribosyl transferase (HPRT) and β-actin. As a positive control we chose the corticosteroid hormone-induced factor (CHIF or FXYD4), a γ-subunit of the Na^+/K^+-ATPase, whose expression level is regulated by aldosterone in colonic epithelium (Wald *et al.* 1996). Initial relative quantities of the genes of interest were normalized to the reference genes. HPRT and β-actin expression levels were similar in all tested groups.

Immunohistochemistry of BK channels

On-slide 10 μm cryostat-slices from non-fixed $BK^{+/+}$ and $BK^{-/-}$ mouse distal colon segments were used. After pre-incubation with 10% normal donkey serum in buffer (1% bovine serum albumin, 0.5% Triton X-100, 0.05 M Tris-buffered saline (TBS)) and rinsing with TBS, the slices were incubated with anti-BK$\alpha_{(674–1115)}$ (1 : 1000 in buffer) and tagged with Alexa 555-conjugated donkey anti-rabbit IgG (1 : 1000 in buffer). This antibody was produced in the laboratory of Peter Ruth (Tübingen, Germany). BK channel immunofluorescence was analysed using a confocal laser-scanning microscope (Biorad MRC1000 attached to Nikon Diaphot 300 and equipped with a krypton–argon laser). For histological analysis of distal colon segments from $BK^{+/+}$ and $BK^{-/-}$ mice the common Masson-Goldner staining method was performed and evaluated using a Leica Aristoplan microscope equipped with a digital camera. No apparent differences were observed (data not shown).

Table 1. Primer and probe sequences and concentrations for qPCR

Gene name	Sequence	Used concentration (nM)
*KCNMA*1 (α)		
Forward primer 5′–3′	CTCCAATGAAATGTACACAGAATATCT	300
Reverse primer 3′–5′	CTATCATCAGGAGCTTAAGCTTCACA	300
Probe	CCTTCGTGGGTCTGTCCTTCCCTACTGTT	200
KCNMB1 (β1)		
Forward primer 5′–3′	TAGTGTGTGCTGCCATCACC	100
Reverse primer 3′–5′	GCTCTTCCTGGTCCTTGATATTAG	300
Probe	CGTCCTGGGTACAACTGTGCTGCC	300
KCNMB2 (β2)		
Forward primer 5′–3′	TGCAGGACCAACATCCTCTAAG	300
Reverse primer 3′–5′	CTTCAGAGCTGTCACAGTTTTCC	300
Probe	TGGACCAGTGGCCGGACCTCTTCA	100
KCNMB3 (β3)		
Forward primer 5′–3′	ACTCGGGACAGAAAGTGCTTC	500
Reverse prime 3′–5′r	GATGTCCAGGACGCTGTTGAG	300
Probe	ACGACGACGAGGCCATCAGGACCA	300
KCNMB4 (β4)		
Forward primer 5′–3′	CAACTCCGAGTCCAACTCCAG	300
Reverse primer 3′–5′	TCTCTTACAGGGCGGGATATAGG	100
Probe	ACACAGCGACCAGCACCAGCTCCT	200
CHIF (corticosteroid hormone-induced factor)		
Forward primer 5′–3′	TGACCCAGTTGATAAAGACAGTCC	300
Reverse primer 3′–5′	CATTTGCACTTGCCACTCAGG	300
Probe	TTCCAGCGATGCACAGGAGCCCTC	200
β-*Actin*		
Forward primer 5′–3′	TTTGCAGCTCCTTCGTTGCC	100
Reverse primer 3′–5′	CACGATGGAGGGGAATACAGC	500
Probe	TCCACACCCGCCACCAGTTCGCC	300
HPRT		
Forward primer 5′–3′	CTTCCTCCTCAGACCGCTTTT	300
Reverse primer 3′–5′	TCTAGGTCATAACCTGGTTCATCA	300
Probe	CGCTAATCACGACGCTGGGACTGC	300

Solutions and chemicals

IBTX was purchased from Latoxan (Valence, France). All other chemicals were supplied from Sigma Aldrich or Merck.

Statistics

The data shown are means ± S.E.M. *n* refers to the number of tissue preparations and only one preparation was used from each animal. Data were tested for normal distribution (Kolmogorov–Smirnov Test) and Student's *t* test was used to compare mean values between the experimental series. Two effects in the same tissue were tested paired and effects obtained from different tissues were tested unpaired. ANOVA was used to allow multiple comparisons (data in Figs 1, 3 and 4). *P* values of < 0.05 were accepted as statistically significant.

Results

High K^+ diet increases K^+ secretion in mouse distal colon

Our preceding publication identified luminal BK channels as mediators of resting and Ca^{2+}-activated K^+ secretion in mouse distal colon (Sausbier *et al.* 2006). Electrogenic K^+ secretion *ex vivo* measured as the Ba^{2+}-sensitive short-circuit current was completely absent in $BK^{-/-}$ mice (Sausbier *et al.* 2006). Here we confirm that Ba^{2+} added to the luminal side of freshly isolated distal colonic mucosa from NMRI mice changed the transepithelial voltage instantly to more lumen-negative values (Fig. 1*A*). In the following experiments the size of the Ba^{2+}-sensitive K^+ secretion was quantified in NMRI mice on a normal diet compared to mice fed a high K^+ diet for 4 days. This manoeuvre is a well-established way to increase ENaC-mediated electrogenic Na^+ absorption

in an aldosterone-dependent fashion (Will *et al.* 1985). As expected, this approach resulted in the induction of a significant amiloride-sensitive I_{sc} (18.3 ± 6.7 μA cm^{-2} in mice on control diet, $n = 7$, *versus* 188.8 ± 48.7 μA cm^{-2} in mice on high K$^+$ diet, $n = 6$, see Supplemental Fig. 2). Plasma aldosterone levels after 4 days on a high K$^+$ diet increased from a resting value of 336 ± 80 to 4625 ± 474 pg ml^{-1} ($n = 7$).

Figure 1*A* shows two original Ussing chamber traces after the addition of luminal amiloride (100 μM), one from a control animal and the other from an animal on a high K$^+$ diet. The basal electrical parameters in normal tissue were: V_{te} -1.9 ± 0.6 mV; R_{te} 33.1 ± 5.7 Ω cm^2 and I_{sc} -62.4 ± 21.6 μA cm^{-2} ($n = 8$). Luminal Ba^{2+} changed V_{te} by -0.2 ± 0.03 mV to more lumen-negative values. The Ba^{2+}-sensitive I_{sc} amounted to 6.7 ± 1.3 μA cm^{-2} (Fig. 1*B*). In animals on a high K$^+$ diet the basal parameters were: V_{te} -0.3 ± 0.5 mV; R_{te} 29.2 ± 2.7 Ω cm^2 and I_{sc} -7.5 ± 21.0 μA cm^{-2} ($n = 6$). Luminal Ba^{2+} changed V_{te} to more lumen-negative values by -0.7 ± 0.2 mV. Under these conditions the Ba^{2+}-sensitive I_{sc} had increased to 26.6 ± 10.9 μA cm^{-2} (Fig. 1*B*). In summary, these results show that electrogenic distal colonic K$^+$ secretion is significantly increased in NMRI mice fed a high K$^+$ diet. This is consistent with known increased faecal K$^+$ excretion in mice treated on a high K$^+$ diet (Arrighi *et al.* 2001).

Ba^{2+}-sensitive K$^+$ secretion is activated by luminal ionomycin

In the following experiment we used luminal ionomycin to investigate Ca^{2+}-activated K$^+$ secretion in NMRI mice fed a high K$^+$ diet. Luminal ionomycin is assumed to preferentially increase subapical $[Ca^{2+}]_i$ in colonic enterocytes (see Discussion). Figure 1*A* shows the effect of luminal stimulation with the Ca^{2+} ionophore ionomycin (1 μM) in the control group as well as in the group on high K$^+$ diet. In control animals addition of luminal ionomycin did not significantly change the transepithelial electrical parameters (V_{te}: -1.0 ± 0.2 *versus* -0.8 ± 0.2 mV; R_{te} 25.4 ± 2.5 *versus* 24.4 ± 2.2 Ω cm^2 and I_{sc} -39.1 ± 8.0 *versus* -30.9 ± 8.5 μA cm^{-2}, $n = 7$). Subsequent inhibition of K$^+$ secretion with luminal Ba^{2+} (5 mM) showed a Ba^{2+}-sensitive I_{sc} of 21.2 ± 5.7 μA cm^{-2} (Fig. 1*B*). In the high K$^+$ diet group, addition of luminal ionomycin induced a significant change of transepithelial voltage to more lumen- positive values from 0.5 ± 0.3 to 1.1 ± 0.3 mV ($n = 6$). Note the positive resting V_{te} values in the K$^+$-loaded animals. R_{te} remained unchanged (26.0 ± 4.0 *versus* 25.3 ± 3.8 Ω cm^2) and I_{sc} changed from 21.3 ± 14.3 to 46.4 ± 14.3 μA cm^{-2}. These animals showed a greatly increased Ba^{2+}-sensitive I_{sc} of 44.5 ± 8.9 μA cm^{-2} (Fig. 1*B*). These results show that luminal ionomycin in mice on a control diet and

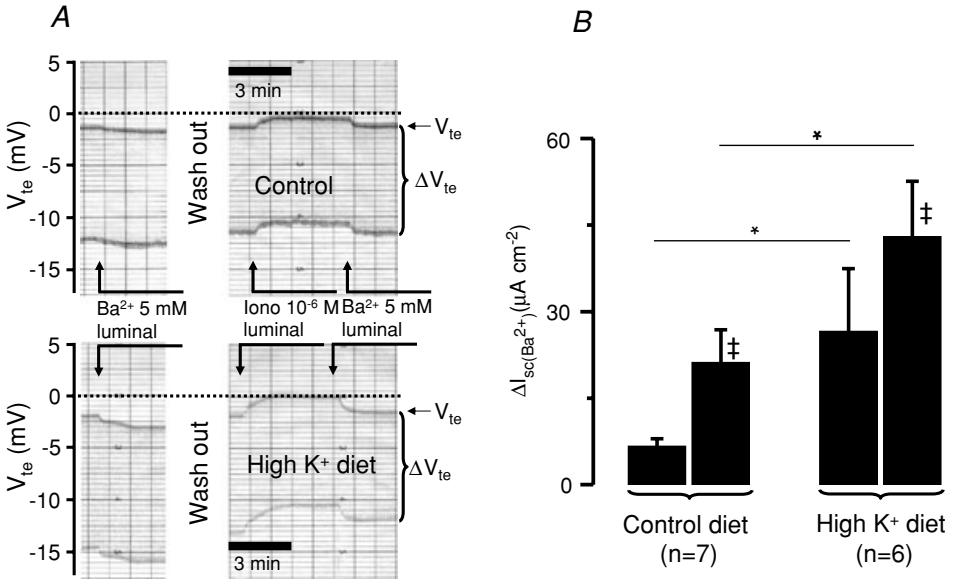

Figure 1. High K$^+$ diet increases Ba^{2+}-sensitive K$^+$ secretion in mouse distal colon
A, two original Ussing chamber traces are depicted, the upper one is from a control animal and the lower from a mouse fed a high K$^+$ diet. The figure shows the resting and luminal Ba^{2+} and the luminal ionomycin-induced transepithelial voltage (V_{te}) changes in the continuous presence of luminal amiloride (100 μM). The upper line indicates V_{te} and the bandwidth of voltage deflections reflects the transepithelial resistance (R_{te}, see Methods). After testing the resting luminal Ba^{2+} effect the tissue was washed and the experiment continued to quantify the Ba^{2+} after addition of luminal ionomycin. *B*, summary of calculated Ba^{2+}-sensitive short-circuit current. *Statistically significant difference between animals of a normal and high K$^+$ diet. ‡Significant differences in the paired controls.

in those fed a high K^+ diet triggered an increase of luminal Ba^{2+}-sensitive I_{sc}. The magnitude of this luminal ionomycin effect is similar in both experimental series. This indicates that the same amount of additional BK channel-dependent current was activated when $[Ca^{2+}]_i$ was increased.

Importantly, $BK^{-/-}$ mice did not show any luminal ionomycin (1 μM)-induced K^+ secretion. Basal parameters in $BK^{+/+}$ mucosa activated with ionomycin were: V_{te} -1.6 ± 0.3 mV; R_{te} $27.3 \pm 0.9\ \Omega\ cm^2$ and I_{sc} $-65.1 \pm 12.8\ \mu A\ cm^{-2}$ ($n = 9$). Luminal Ba^{2+} changed V_{te} by -0.7 ± 0.1 mV and I_{sc} by $24.8 \pm 2.3\ \mu A\ cm^{-2}$. R_{te} remained unchanged ($0.00 \pm 0.52\ \Omega\ cm^2$). In $BK^{-/-}$ preparations electrical values before ionomycin were: V_{te} -6.0 ± 1.4 mV; R_{te} $52.7 \pm 9.2\ \Omega\ cm^2$; and I_{sc} $-127.6 \pm 27.5\ \mu A\ cm^{-2}$ ($n = 5$) and luminal ionomycin had no effect (V_{te} -6.0 ± 1.4 mV; R_{te} $52.0 \pm 9.0\ \Omega\ cm^2$; and I_{sc} $-128.4 \pm 28.7\ \mu A\ cm^{-2}$). These data indicate that the K^+ diet-induced up-regulated luminal K^+ conductance can be activated by an elevation of intracellular Ca^{2+}.

K^+ loading increases luminal iberiotoxin-sensitive K^+ secretion

In the context of our previous study these results strongly imply that the increased K^+ secretion in animals on a high K^+ diet occurs via increased activity of luminal BK channels. To investigate this pharmacologically the following experiments were performed to quantify the iberiotoxin-sensitive K^+ secretion in control and K^+-loaded animals. IBTX is a specific BK channel blocker (Galvez *et al.* 1990). IBTX-sensitive short-circuit current is taken as a measure of BK channel-mediated K^+ secretion. Figure 2*A* shows the effect of luminal IBTX (240 nM) on the transepithelial voltage. The effect of luminal IBTX compared to luminal Ba^{2+} is very slow, probably because the diffusion of IBTX to its site of action is impeded by the mucous layer or the complex geometry of the infolded epithelium. Luminal IBTX changed V_{te} by -0.3 ± 0.1 mV measured after 20 min in controls ($n = 6$) and -0.8 ± 0.1 mV in K^+-fed animals ($n = 6$). These voltage changes correspond to changes in current by $14.9 \pm 2.6\ \mu A\ cm^{-2}$ and $31.8 \pm 5.9\ \mu A\ cm^{-2}$, respectively (Fig. 2*B*). The summary of the IBTX experiments (Fig. 2*B*) clearly shows that the IBTX-sensitive short circuit current is greatly increased in colonic mucosa from K^+-loaded mice. Note that IBTX had no effect on V_{te} when applied to the luminal surface of $BK^{-/-}$ mouse preparations (Sausbier *et al.* 2006).

Absence of Ba^{2+}-sensitive K^+ secretion in K^+-loaded $BK^{-/-}$ mice

The increased K^+ secretion on a high K^+ diet could be caused by induction of the existing BK channel or alternatively the introduction of a new luminal K^+ conductance. Therefore, we investigated the Ba^{2+}-sensitive I_{sc} in $BK^{+/+}$ and $BK^{-/-}$ mice treated on a high K^+ diet for 4 days. Tissue resistances between the two genotypes were not different ($BK^{+/+}$: $51.9 \pm 3.7\ \Omega\ cm^2$, $BK^{-/-}$: $45.6 \pm 2.1\ \Omega\ cm^2$). As reported previously (Sausbier *et al.* 2006), the resting transepithelial voltages were more lumen-negative in $BK^{-/-}$ mice ($BK^{+/+}$: -1.6 ± 0.3 mV, $BK^{-/-}$: -3.8 ± 0.7 mV). The effectiveness of high K^+ treatment can be seen as induction of amiloride-sensitive I_{sc}. $BK^{-/-}$ mice under a normal diet have an increased plasma aldosterone level (Sausbier *et al.* 2005) and this can be indirectly seen as an increased colonic amiloride-sensitive I_{sc} (Sausbier *et al.* 2006). We confirm these data and find that $\Delta I_{sc(amil)}$ was $16.4 \pm 3.1\ \mu A\ cm^{-2}$ in $BK^{+/+}$ ($n = 8$) *versus* $46.5 \pm 14.8\ \mu A\ cm^{-2}$ in $BK^{-/-}$ ($n = 5$). After 4 days of a high K^+ diet, $BK^{+/+}$ mice showed a large increase of $\Delta I_{sc(amil)}$ to $179.0 \pm 40.7\ \mu A\ cm^{-2}$ ($n = 8$) and $BK^{-/-}$ mice present a dramatic increase of $\Delta I_{sc(amil)}$ to $394.3 \pm 97.5\ \mu A\ cm^{-2}$ ($n = 8$, see

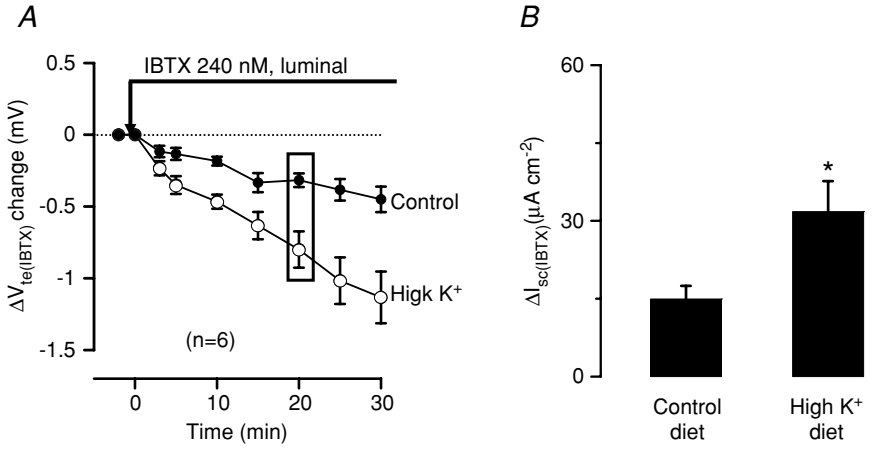

Figure 2. K^+ loading increases luminal iberiotoxin-sensitive K^+ secretion
Luminal iberiotoxin (IBTX) slowly changed the transepithelial voltage (V_{te}) to more lumen-negative values. *A* is a summary of 6 experiments. *B* displays the corresponding changes of short-circuit current calculated 20 min after addition of IBTX. Note that the high K^+ diet augmented the IBTX-sensitive short-circuit current.

Supplemental Fig. 2). This has to be viewed in the light of a dramatically increased plasma aldosterone in $BK^{-/-}$ mice on a high K^+ diet (Rieg *et al.* 2007). Importantly, $BK^{-/-}$ mice on a high K^+ diet do not show a luminal Ba^{2+}-sensitive I_{sc} (Fig. 3). These results strongly indicate that in the presence of elevated plasma aldosterone no alternative Ba^{2+}-sensitive luminal K^+ conductance has appeared. They support the idea that the BK channel is the relevant luminal K^+ secretory channel in mouse distal colon.

Semi-quantitative PCR analysis of BK channel gene transcripts

Since aldosterone is a strong modulator of gene expression it is likely that the observed functional effects are caused by transcriptional up-regulation of the relevant K^+ channel mRNAs. Therefore, we investigated if a high K^+ diet affects the mRNA levels of the α-subunit and the related four β-subunits of the BK channel (KCNMA1, $K_{Ca}1.1$). As a positive control we tested the known aldosterone-induced gene corticosteroid hormone-induced factor (*CHIF*, *FXYD4*) (Wald *et al.* 1996). The genes of interest were compared to the reference genes *β-actin* and *HPRT*. The strong up-regulation (8-fold) of CHIF in NMRI mice on a high K^+ diet confirmed the strength of the method. K^+-loaded animals show a relative 2-fold increase in mRNA expression of the pore-forming BK α-subunit (KCNMA) and the auxiliary BK β_2-subunit (KCNMB2) ($n = 7$). These 2-fold increases of α- and β_2-subunit mRNA were identical when compared to both reference genes (Fig. 4). Both, the β_1- and β_4-subunits showed very low expression in the control colonic preparations and the β_3-subunit could not be detected at all. None of these genes changed their expression level after K^+ loading.

Immunolocalization of BK channels

Figure 5 shows the localization of BK channel α-subunits in mouse distal colonic mucosa on a normal diet and after a high K^+ diet. The green colour represents intrinsic autofluorescence and provides a crude morphological image of the colonic tissue. The red stain reflects localization of BK channels and the specificity is ensured by the complete lack of staining in $BK^{-/-}$ tissue (Fig. 5*E*). Under the normal diet weak staining is visible at the luminal membrane domain of crypt cells (Fig. 5*A*, arrowheads). Animals on a high K^+ diet show a marked increase of the staining associated with the luminal membrane of crypt enterocytes (Fig. 5*B*, *D* and *F*; see arrowheads). Unexpectedly, animals on a high K^+ diet show strong staining in the tissue surrounding the colonic crypts (Fig. 5). The anatomical structure of this staining remains to be defined. It should be noted that distinct staining of basolateral membrane domains can be seen in Fig. 5*B* and *F*, which has newly appeared in mice treated with a high K^+ diet.

Exogenous aldosterone increases colonic K^+ secretion in a spironolactone- and iberiotoxin-sensitive manner

In the following experiments we investigated if aldosterone added *ex vivo* to freshly isolated colonic mucosa is able to mimic the effect of a high K^+ diet. Electrogenic K^+ transport was measured directly (short circuit) as the Ba^{2+}-sensitive I_{sc}. The size of Ba^{2+}-sensitive K^+ secretion

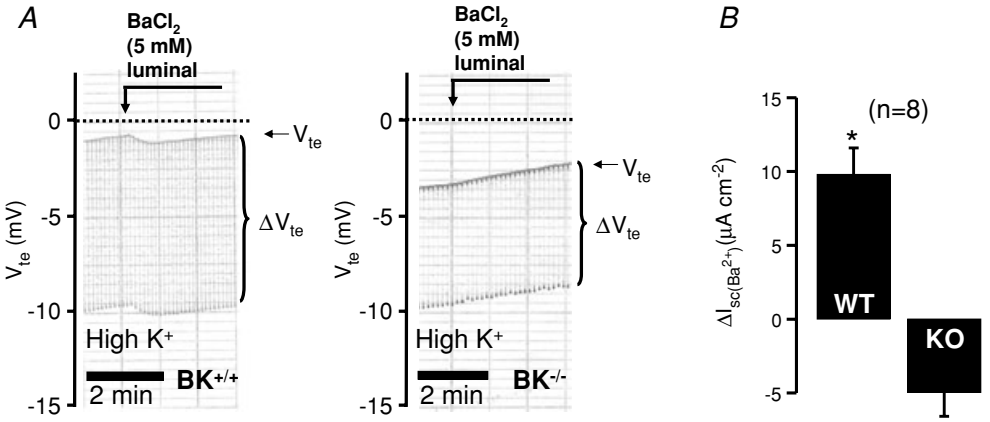

Figure 3. Luminal Ba^{2+}-induced changes of transepithelial voltage in distal colon from $BK^{+/+}$ and $BK^{-/-}$ mice treated on a high K^+ diet for 4 days
A shows resting and luminal Ba^{2+}-induced V_{te} changes in the continuous presence of luminal amiloride (100 μM). The upper line indicates V_{te} and the bandwidth of voltage deflections reflects the transepithelial resistance (R_{te}, see Methods) *B*, summary of calculated Ba^{2+}-sensitive short-circuit current. WT, wild-type; KO, knock-out mice. *Statistically significant differences between animals of normal and high K^+ diet.

was quantified in distal colon before and after 2 h of aldosterone incubation. The resting Ba^{2+}-sensitive I_{sc} in freshly isolated tissue was $4.9 \pm 2.1\ \mu A\ cm^{-2}$ and amounted to $3.2 \pm 1.8\ \mu A\ cm^{-2}$ ($n = 8$) after 2 h in the time control experiments (Fig. 6). A concentration of 1 nM aldosterone was not enough to increase K^+ secretion after 2 h; however, it appeared to counteract the decay in K^+ secretion seen in the time controls. The values were $5.4 \pm 1.4\ \mu A\ cm^{-2}$ before and $5.6 \pm 2.2\ \mu A\ cm^{-2}$ ($n = 7$) after the incubation period (Fig. 6). Incubation with 10 nM aldosterone resulted in an increase of Ba^{2+}-sensitive I_{sc} to $10.6 \pm 1.5\ \mu A\ cm^{-2}$ ($n = 10$). The same results were seen with an aldosterone concentration of 100 nM ($11.6 \pm 2.5\ \mu A\ cm^{-2}$; $n = 7$; Fig. 6).

Subsequently, we investigated if the effect of aldosterone was mediated via mineralocorticoid receptors (MRs). In the presence of spironolactone (1 μM), 10 nM aldosterone no longer induced K^+ secretion. The Ba^{2+}-sensitive I_{sc} was not changed over the 2 h incubation ($6.1 \pm 1.0\ \mu A\ cm^{-2}$ *versus* $4.3 \pm 0.8\ \mu A\ cm^{-2}$, $n = 7$, Fig. 7). This increase in Ba^{2+}-sensitive K^+ secretion occurred via BK channels because IBTX (120 nM) prevented the 10 nM aldosterone-induced I_{sc} increase. The initial Ba^{2+}-sensitive I_{sc} was $8.0 \pm 0.8\ \mu A\ cm^{-2}$ and after the incubation it had changed to $4.4 \pm 1.2\ \mu A\ cm^{-2}$ ($n = 8$, Fig. 7). These results show that high K^+ diet and exogenously added aldosterone resulted in a similar K^+ handling phenotype in mouse distal colon. This K^+ secretion is inhibited by MR receptor blockage.

Discussion

Aldosterone increases colonic K^+ secretion via up-regulation of luminal BK channel

Colonic K^+ handling is one determinant of whole-body K^+ homeostasis. Net colonic K^+ handling is defined by the balance of K^+ absorption via the luminal colonic H^+/K^+-ATPase and the amount of secreted K^+. The ion channel responsible for K^+ secretion in the distal colon was recently defined as the Ca^{2+}-activated BK channel in the luminal membrane of colonic crypt epithelium. $BK^{-/-}$ mice showed neither resting nor $[Ca^{2+}]_i$-stimulated luminal K^+ secretion, which was reflected in reduced faecal

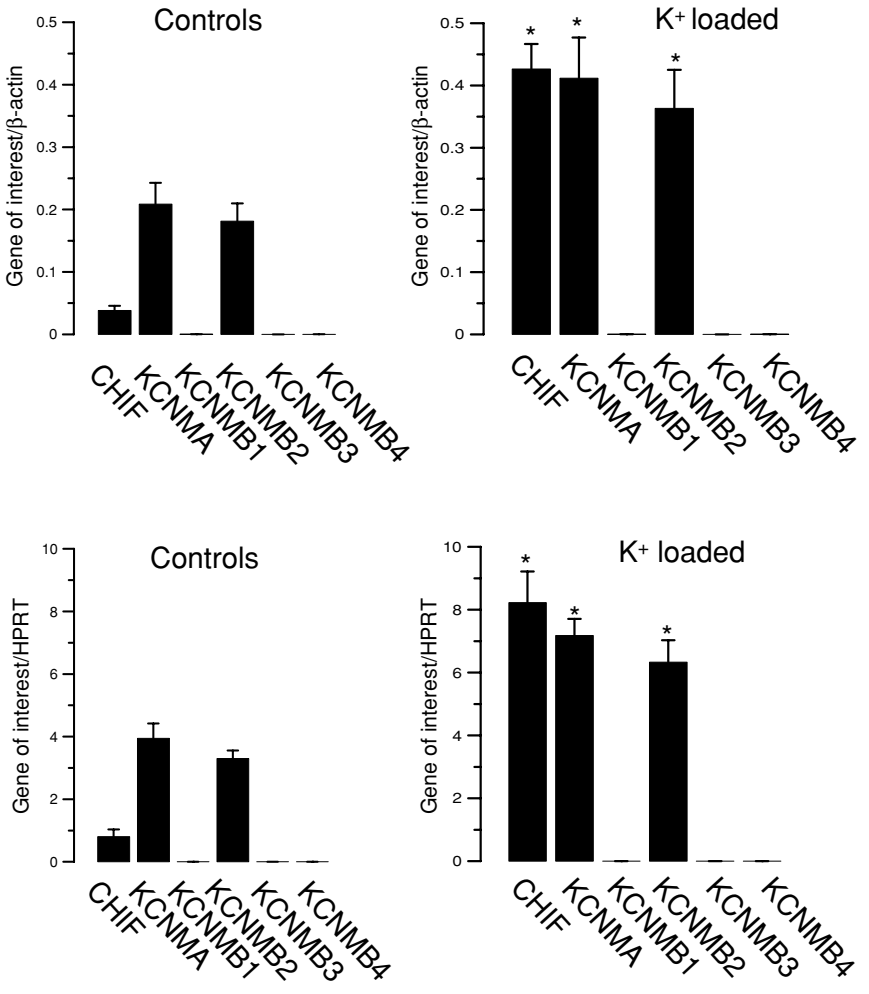

Figure 4. K^+ loading increases the mRNA expression levels of BK α- and β_2-subunits in isolated colonic crypts (from 7 male animals in each group)
Relative expression levels of mRNA of the genes of interest are shown in comparison to β-actin (upper panels) and to HPRT (lower panels). Control data are shown in the left panels and those obtained from K^+-loaded mice on the right. Corticosteroid hormone-induced factor (CHIF) served as a positive control for the action of aldosterone. *Statistically significant difference ($P < 0.05$) between groups.

K^+ excretion (Sausbier *et al.* 2006). Colonic K^+ secretion is known to be activated by aldosterone (Pacha *et al.* 1987; Sweiry & Binder, 1989; Rechkemmer & Halm, 1989; Halm & Halm, 1994; Binder & Sandle, 1994). Recently, it was found that knock-out mice for the regulatory KCNE1 subunit of the voltage-dependent I_{Ks} K^+ channel show significantly elevated plasma aldosterone levels (Arrighi *et al.* 2001). Interestingly, these mice display profound hypokalaemia and significant faecal K^+ wasting (Arrighi *et al.* 2001). These results support the idea that aldosterone is also an important activator of intestinal K^+ excretion in the mouse.

In this study we asked if aldosterone-induced K^+ secretion occurs via BK channels. Several lines of evidence support this conclusion: (1) a high K^+ diet led to a 2-fold increase of the luminal Ba^{2+}- and IBTX-sensitive I_{sc} in distal mouse colonic mucosa; (2) a high K^+ diet in $BK^{-/-}$ mice failed to induce a Ba^{2+}-sensitive K^+ secretion; (3) the resting and diet-induced enhanced K^+ secretion is stimulated by luminal ionomycin; (4) in $BK^{-/-}$ mice luminal ionomycin was completely ineffective in stimulating K^+ secretion; (5) *in vitro* addition of aldosterone likewise triggered a 2-fold increase in K^+ secretion which was sensitive to the MR blocker spironolactone and IBTX; (6) semi-quantitative RT-PCR from isolated colonic crypts showed significant up-regulation of BK α- and β_2-subunit mRNA; (7) BK immunohistochemistry confirmed the previous results of the luminal localization in colonic crypt cells and showed that this luminal staining of BK channels is strongly up-regulated in K^+-loaded animals. These combined results define that the luminal BK channel is the target of the aldosterone-induced colonic K^+ secretion and it is suggested that this occurs via the classic action of aldosterone through its mineralocorticoid receptor.

The nature and localization of the K^+ secretory channel in distal colon

The nature of the K^+ secretory channel in colonic mucosa was until recently a matter of debate. Several candidates had been suggested including the SK, IK and BK potassium channels (Butterfield *et al.* 1997; Joiner *et al.* 2003) and, in addition, many other K^+ channels known to be expressed luminally in other segments of the gastrointestinal tract could be putative players (Warth & Barhanin, 2003). The notion that the BK channel is the relevant candidate originated from the following observations: (1) BK channels are present in the luminal membrane of mammalian K^+ secretory epithelia (Hunter *et al.* 1984; Woda *et al.* 2001; Frindt & Palmer, 2004), and (2) BK channels are found in isolated surface enterocytes from rat distal colon (Butterfield *et al.* 1997). Our study

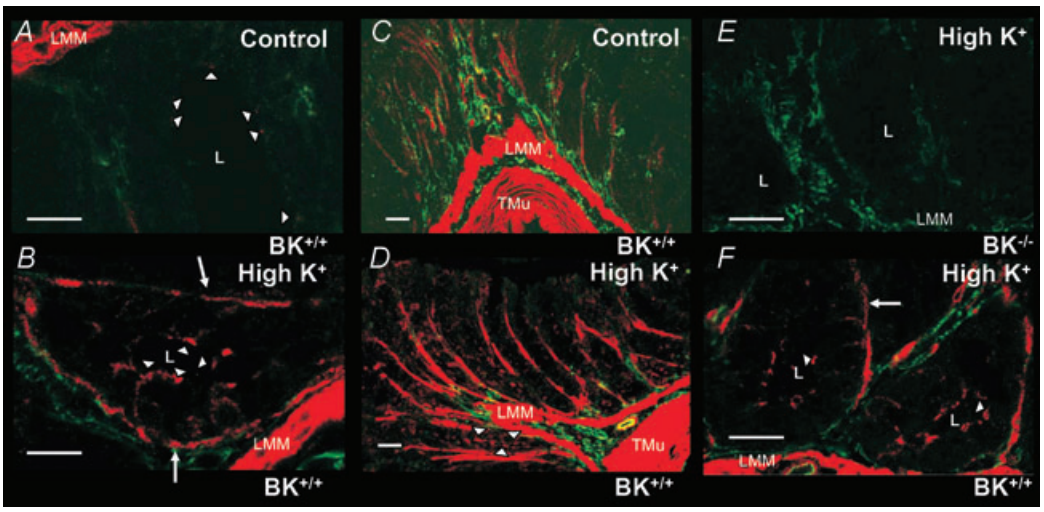

Figure 5. Immunohistochemical localization of BK channel α-subunits in mouse distal colon under normal and high K^+ diet

The green colour represents intrinsic autofluorescence providing crude morphological orientation. The red stain reflects localization of BK channel α-subunits and is specific as indicated by the complete absence of staining in $BK^{-/-}$ tissue (*E*). Under normal diet, red staining is faintly visible in the luminal membrane domain of crypt cells (*A*, arrowheads). Animals on a high K^+ diet show a marked increase of red staining associated with the luminal membrane of crypt enterocytes (*B*, *D* and *F*; arrowheads). Mucosa from animals on a high K^+ diet showed additional enhanced staining in the tissue surrounding the colonic crypts (*D*). The anatomical structure of this remains to be established. Note that novel BK staining associated with the basolateral membrane domains can be seen in high K^+-treated tissues (arrows, *B* and *F*). Scale bars in *A*, *B*, *E* and *F*: 25 μm; *C* and *D*: 50 μm. Abbreviations: L, crypt lumen; LMM, laminal muscularis mucosae; TMu, tunica muscularis mucosae.

using the $BK^{-/-}$ mouse provided strong evidence that this channel is the crucial player in mouse distal colonic K^+ secretion (Sausbier *et al.* 2006). This paper also presented evidence that leaves little room for other luminal K^+ channels relevant for colonic K^+ secretion. However, controversial data exist with regard to the localization of the BK channel along the crypt-to-surface epithelial axis, with several immunolabelling studies supporting BK expression in surface epithelial cells (Grunnet *et al.* 1999; Hay-Schmidt *et al.* 2003; Sandle *et al.* 2007; Puntheeranurak *et al.* 2007; Flores *et al.* 2007). In sharp contrast, our preceding results present evidence that luminal BK channels preferentially localize to the crypt epithelium and to a much lesser degree to surface enterocytes (Sausbier *et al.* 2006). The current immunohistochemical data support the idea that K^+ loading does not promote a strong expression in surface cell luminal membranes. Noteworthy, our immunolocalization studies of BK channels in colonic epithelium are currently the only ones available with the important negative control performed in $BK^{-/-}$ tissue. Active K^+ secretion can be inhibited by interfering with the basolateral K^+ uptake step with either ouabain to inhibit the Na^+/K^+-ATPase or with loop diuretics to block NKCC1 (Sweiry & Binder, 1989; Rechkemmer *et al.* 1996). NKCC1 is localized preferentially to the basolateral membranes of the crypt and not to surface colonic epithelium (Pena-Munzenmayer *et al.* 2005). In summary, this supports the idea that BK channel-dependent K^+ secretion is a prominent function of colonic crypts and not surface cells. An unexpected finding demonstrated in Fig. 5*B* and *F* indicates that a high K^+ diet might provoke BK channel expression in the basolateral membrane of colonic crypt cells. Interestingly, we have recently found that BK channels are

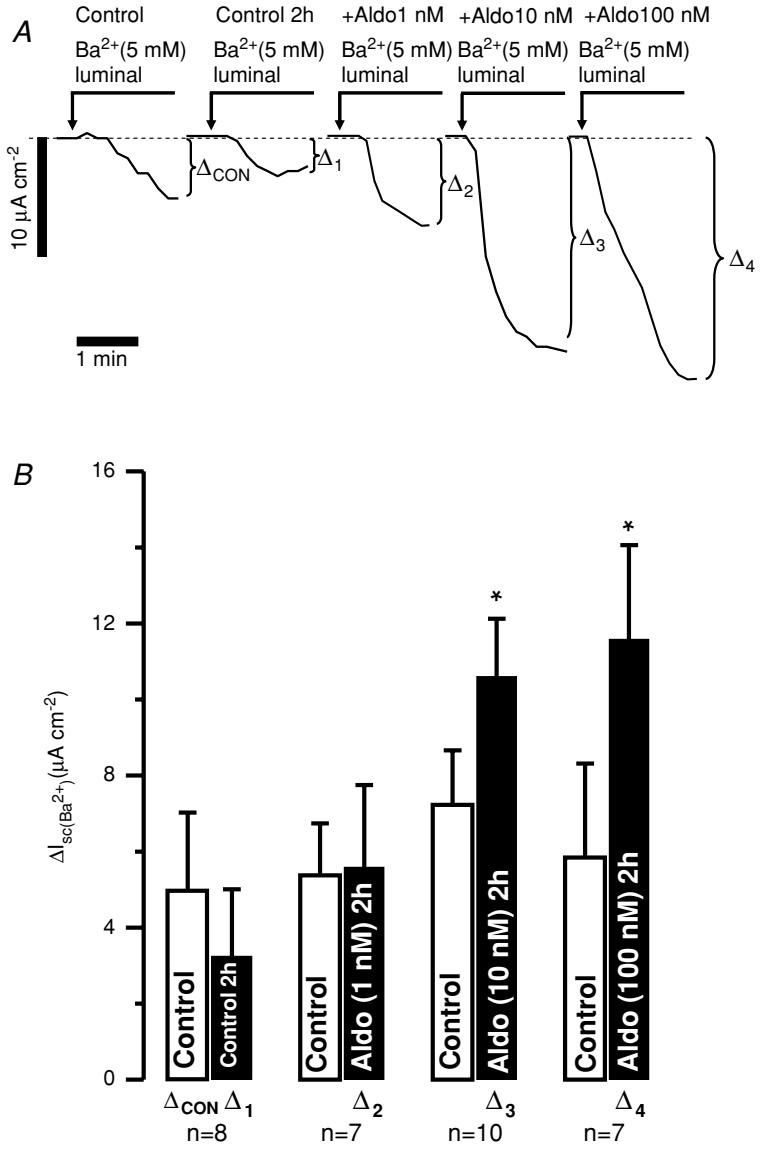

Figure 6. Exogenous aldosterone increases Ba^{2+}-sensitive I_{sc} in freshly isolated mouse distal colonic mucosa
A, representative original traces from different experiments of luminal Ba^{2+}-induced changes of I_{sc} in time control after 2 h (Δ_1) and in tissues treated for 2 h with increasing aldosterone (Aldo) concentrations (1 nM, Δ_2; 10 nM, Δ_3 and 100 nM, Δ_4). *B*, summary. *Statistical significance ($P < 0.05$) compared to the untreated time controls (2 h).

not functionally relevant for cholinergic (via intracellular Ca^{2+})-stimulated anion secretion in colon from animals on a normal diet (Matos *et al.* 2007). The above-mentioned data indicate that basolateral BK channels may become functionally relevant for $[Ca^{2+}]_i$-activated anion secretion under high K^+ diet treatment. This needs to be further investigated.

BK channel-mediated K^+ secretion in other secretory epithelia

A comparative view towards other epithelia provides the intriguing observation that K^+-secretory tissues in general use this ion channel as one relevant luminal K^+ exit pathway. There is circumstantial evidence for this in salivary glands (Romanenko *et al.* 2007) and several studies highlight the role of BK channels in renal flow-stimulated K^+ secretion (Woda *et al.* 2001; Bailey *et al.* 2006). In addition to the fundamental role of ROMK channels for renal K^+ secretion under basal conditions (Hebert *et al.* 2005) BK channel-mediated renal K^+ secretion works in parallel and becomes active under high tubular flow conditions (Woda *et al.* 2001; Bailey *et al.* 2006; Pluznick & Sansom, 2006). BK channels are composed of the pore-forming α-subunit and one associated β-subunit (β_{1-4}). The β-subunit renders the channel functional under physiological membrane voltages and $[Ca^{2+}]_i$ concentrations when coexpressed with the α-subunit. It is therefore believed that functional BK channels form hetero-octamers composed of four α-subunits and four

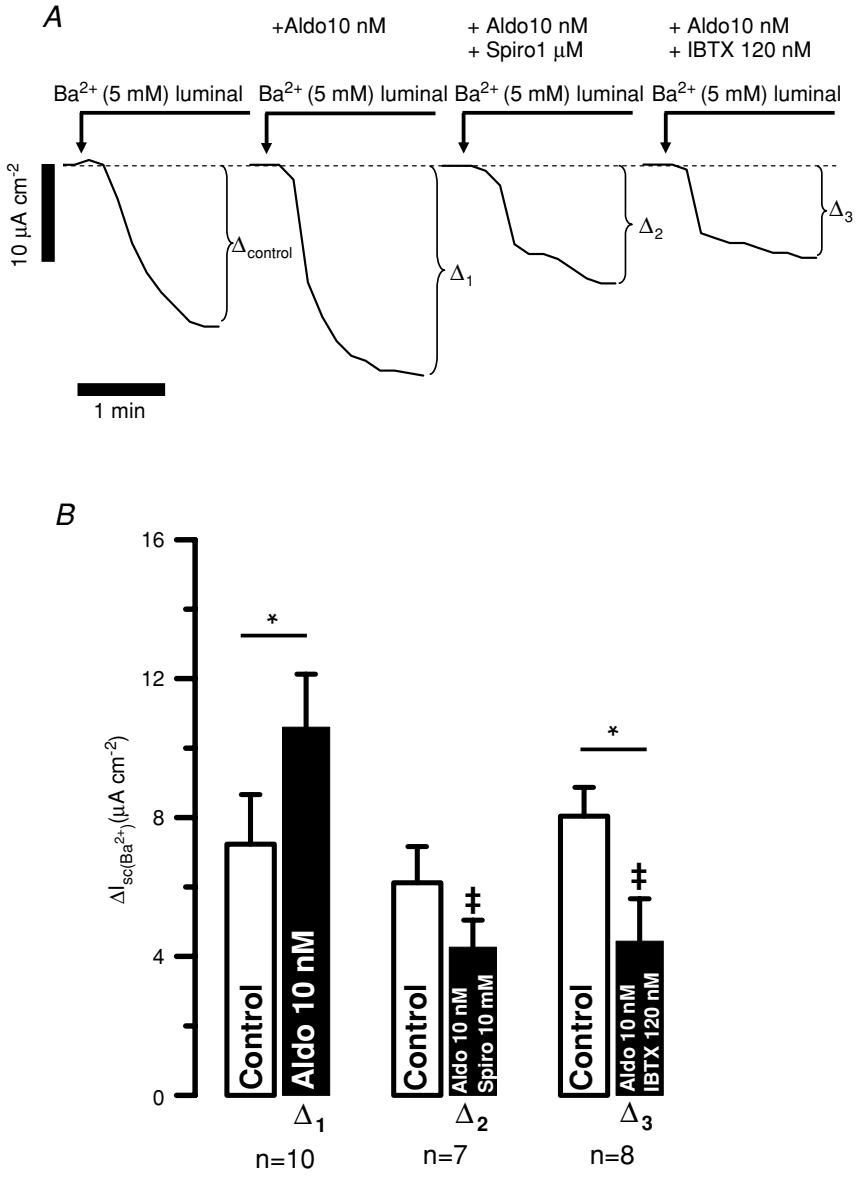

Figure 7. The Ba^{2+}-sensitive I_{sc} increased by exogenous aldosterone was inhibited by spironolactone (Spiro, 1 μM) and IBTX (120 nM) in freshly isolated mouse distal colonic mucosa
A, representative original traces of different luminal Ba^{2+}-induced changes of I_{sc} in control, in aldosterone (10 nM, Δ_1), in aldosterone (10 nM) plus spironolactone (1 μM, Δ_2) and in aldosterone (10 nM) plus IBTX (120 nM, Δ_3)-treated tissues. *B*, summary. ‡Statistical significance compared to tissue treated with aldosterone only ($P < 0.05$). *Statistical significance ($P < 0.05$) compared to the untreated pre-controls.

associated β-subunits (Salkoff *et al.* 2006; Torres *et al.* 2007). We find clear evidence that the associated regulatory β-subunit in colonic enterocytes is β_2. Also mRNA of this protein is up-regulated *in vivo* under conditions of high aldosterone. Intriguingly, the β_2-subunit causes rapid inactivation of the human BK channel (Wallner *et al.* 1999). In mouse distal colon luminal nucleotides via luminal $P2Y_2$ or $P2Y_4$ receptors cause rapid and transient K^+ secretory bursts (Matos *et al.* 2005; Sausbier *et al.* 2006). It is therefore tempting to speculate that this transient K^+ secretion could relate to β_2-subunit-mediated fast deactivation of BK channels. Alternatively, the transient K^+ secretory burst could merely mirror a fast and transient subapical $[Ca^{2+}]_i$ transient.

Our data are in agreement with semi-quantitative RT-PCR data from microdissected rabbit cortical collecting ducts which indicate up-regulation of α- and β_2-subunits in animals on a high K^+ diet (Najjar *et al.* 2005). In that study the β_3- and β_4-subunits were also found to be up-regulated (Najjar *et al.* 2005), an observation we had not seen in distal colonic mucosa. In renal epithelia the issue of the associated β-subunits in the K^+-secretory distal tubule continues to be complex as has been reviewed recently (Pluznick & Sansom, 2006). The β_1-subunit has been suggested to be functionally relevant for connecting tubule high flow-induced K^+ secretion (Pluznick *et al.* 2005) and that the β_4-subunit may be critical for collecting duct-mediated K^+ secretion (Grimm *et al.* 2007). Further studies are necessary to outline which BK β-subunits are present and functionally relevant in renal and other K^+-secretory epithelia.

Time course of up-regulated K^+ secretion in colonic epithelium

Mineralocorticoid action on Na^+- and K^+-transporting epithelia can be divided into early (1.5–3 h) and late (6 h to days) responses (Verrey, 1995, 1999). Non-genomic, very fast aldosterone actions are not discussed here. Transcriptional regulation is the underlying cause of both the 'regulatory' fast effects and the 'anabolic-type' late effects (Verrey, 1999). Numerous aldosterone-induced and repressed genes are identified today, some of which show expression changes in different aldosterone-sensitive model epithelia within the first 30 min (e.g. SGK1) (Naray-Fejes-Toth & Fejes-Toth, 2000; Robert-Nicoud *et al.* 2001). Colonic up-regulation of ENaC-mediated Na^+ absorption is an example of a late aldosterone response (Epple *et al.* 2000). In contrast, the effect of aldosterone on the distal colonic K^+ secretion reaches maximal values within the first 2 h, which clearly preceded its effect on Na^+ transport (Halm & Halm, 1994). In our study aldosterone stimulated colonic K^+ secretion *in vitro* 2-fold after 2 h, which was similar in magnitude to the increase observed after feeding a high K^+ diet for 4 days. The fast aldosterone response was completely inhibited with spironolactone demonstrating that transcriptional regulation is involved. The protein and mRNA level of the BK channel proteins were not investigated after 2 h of aldosterone treatment. The cause of the rapid up-regulation of BK channel function remains to be elucidated.

In summary, this study extends our previous finding that BK channels are the sole luminal K^+ ion channel relevant for colonic K^+ secretion. The aldosterone-induced increase of electrogenic K^+ secretion occurs via increased abundance of BK channels in the luminal membrane of colonic crypts and not in colonic surface cells.

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No conflict of interest exists.

Supplemental material

Online supplemental material for this paper can be accessed at: http://jp.physoc.org/cgi/content/full/jphysiol.2008.156968/DC1